IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF:
Takashi Shinohara et al.
SERIAL NO.: 10/594,864 : GROUP ART UNIT: 1632
FILED: November 30, 2006 : EXAMINER: Magdalene K. Sgagias
FOR: PROCESS FOR PRODUCING MULTIPOTENTIAL STEM CELL ORIGINATING IN TESTOID CELL

TRANSLATOR'S DECLARATION

Honorable Commissioner for Patents
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Sir:

I, Ritsuko Arimura, declare:

That I am well acquainted with both the Japanese and English languages;

That the attached document represents a true English translation of Japanese Patent Application No. 2004-101320 filed on March 30, 2004; and

That I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 18th day of July, 2008.

Ritsuko Arimura

(Translation)

J A P A N P A T E N T O F F I C E

This is to certify that the annexed is a true copy of the following application as filed with this Office.

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Applicant(s) : Kyoto University

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【List of the Annexed Documents】

【Document】 Claims	One copy
【Document】 Specification	One copy
【Document】 Drawing	One copy
【Document】 Abstract	One copy

【Document】 Claims

【Claim 1】 A method of producing pluripotent stem cells, which comprises culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells.

【Claim 2】 The production method of claim 1, wherein the medium further contains leukemia inhibitory factor (LIF).

【Claim 3】 The production method of claim 1 or 2, wherein the medium further contains at least one of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF).

【Claim 4】 The production method of any one of claims 1 to 3, which comprises culturing testis cells in the presence of feeder cells.

【Claim 5】 The production method of claim 1, which comprises the following steps:

(Step 1) culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain cultured cells;

(Step 2) culturing the cultured cells obtained in Step 1, using a medium containing leukemia inhibitory factor (LIF) to obtain pluripotent stem cells.

【Claim 6】 The production method of claim 5, wherein the medium for Step 1 further contains leukemia inhibitory factor (LIF).

【Claim 7】 The production method of claim 5 or 6, wherein the medium for Step 1 further contains at least one of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF).

【Claim 8】 The production method of any one of claims 5 to 7, wherein Step 1 comprises culturing testis cells in the presence of feeder cells.

【Claim 9】 The production method of any one of claims 1 to 8, wherein the testis cells are derived from a mammal.

【Claim 10】 The production method of claim 9, wherein the mammal is postnatal.

【Claim 11】 The production method of claim 1, wherein the pluripotent stem cells are positive for at least any one

selected from the group consisting of SSEA-1, Forsman antigen, β1-integrin, α6-integrin, EpCAM, CD9, EE2 and c-kit.

【Claim 12】 The production method of claim 11, wherein the pluripotent stem cells are positive for SSEA-1, Forsman antigen, β1-integrin, α6-integrin, EpCAM, CD9, EE2 and c-kit.

【Claim 13】 A pluripotent stem cell produced by the production method of any one of claims 1 to 12.

【Claim 14】 A pluripotent stem cell derived from a testis cell, which is positive for at least any one selected from the group consisting of SSEA-1, Forsman antigen, β1-integrin, α6-integrin, EpCAM, CD9, EE2 and c-kit.

【Claim 15】 The pluripotent stem cell of claim 14, which is positive for SSEA-1, Forsman antigen, β1-integrin, α6-integrin, EpCAM, CD9, EE2 and c-kit.

【Claim 16】 A method of producing a chimeric embryo, which comprises the following steps:

(Step 1) culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells;

(Step 2) introducing the pluripotent stem cells into a host embryo to obtain a chimeric embryo.

【Claim 17】 A method of producing a chimeric animal (excluding humans), which comprises the following steps:

(Step 1) culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells;

(Step 2) introducing the pluripotent stem cells into a host embryo to obtain a chimeric embryo;

(Step 3) transferring the chimeric embryo to the uterus of a host animal to obtain a chimeric animal (excluding humans).

【Claim 18】 A method of producing mesodermal cells, which comprises the following steps:

(Step 1) culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells;

(Step 2) culturing the pluripotent stem cells under mesodermal cell differentiation conditions to obtain mesodermal cells.

【Claim 19】 The production method of claim 18, wherein the mesodermal cells are any one selected from the group consisting of blood cell lineage cells, vascular lineage cells and myocardial cells.

【Claim 20】

A method of producing neuronal lineage cells, which comprises the following steps:

(Step 1) culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells;

(Step 2) culturing said pluripotent stem cells under neuronal lineage cell differentiation conditions to obtain neuronal lineage cells.

【Claim 21】 The method of claim 20, wherein the neuronal lineage cells are neurons or glial cells.

【Claim 22】 A composition for producing pluripotent stem cells derived from a testis cell, which contains glial cell derived neurotrophic factor (GDNF) or an equivalent thereto.

【Claim 23】 The composition of claim 22, which further contains leukemia inhibitory factor (LIF).

【Claim 24】 The composition of claim 22 or 23, which further contains at least one of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF).

【Document】 Specification

【Title of the Invention】 Process for Producing Multipotential Stem Cell Originating in Testoid Cell

【Technical Field of the Invention】

The present invention relates to a method of producing pluripotent stem cells using testis cells, pluripotent stem cells produced by the method, a method of producing a chimeric embryo using said pluripotent stem cells, a method of producing a chimeric animal using said chimeric embryo, a method of producing functional cells such as mesodermal cells from the pluripotent stem cells, a composition for producing pluripotent stem cells derived from testis cells, and the like.

【Background Art】

Germ cells are unique in that they have the capacity to contribute genes to the offspring. Although they are highly specialized cells to make gametes for reproduction, several lines of evidences suggest their multipotentiality. For example, teratomas are tumors that nearly always occur in the gonads, and contain many kinds of cells and tissues in various stages of maturation. Furthermore, fetal germ cells are known to give rise to pluripotential cells when cultured under special condition. These embryonic germ (EG) cells have a differentiation property similar to embryonic stem (ES) cells, isolated from inner cell mass. While these observations strongly suggest that the germline lineage may keep the ability to generate pluripotential cells, it has not been possible to establish pluripotent cells from normal postnatal gonads. Because both ES cells and EG cells are collected from prenatal embryos or fetuses, clinical applications thereof to humans pose a major ethical problem, and there has been a demand for the development of a technology for establishing a pluripotent cell from a postnatal individual.

The present inventors have developed a method of the in vitro culture of mouse spermatogonial stem cell, the only stem cell type in the body that can transmit genetic information to

offspring (Non-patent Reference 1). When neonatal testis cells were cultured in the presence of glial cell derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and the like, germ cells developed uniquely shaped colonies, and stem cells proliferated over five-months period in a log-scale. Upon transplantation into seminiferous tubules of infertile mice, the cultured cells produced normal sperm and offspring, but no teratoma or somatic cell differentiation was observed, indicating that they are fully committed to the germ cell lineage. This was in contrast to ES cells, which produce teratoma after transferring into seminiferous tubules. Based on these results, we have named these cells, germline stem, or GS, cells to distinguish them from ES or EG cells. Thus, GS cells represent a third method to expand germline cells, but are clearly different from ES/EG cells in their differentiation capacity.

[Non-patent Reference 1] Biology of Reproduction, vol. 69, pages 612-616, 2003

[Disclosure of the Invention]

[Problems to be Solved by the Invention]

In view of the above-described circumstances, it is an object of the present invention to provide a new method of producing a pluripotent stem cell from a postnatal individual.

[Means of Solving the Problems]

The present inventors diligently investigated to accomplish the above-described object and confirmed that when newborn mouse testis cells were cultured under conditions similar to those for GS cell culture, colonies morphologically indistinguishable from ES cell colonies emerge in addition to colonies of GS cells. These ES-like cells grew selectively under ES cell culture conditions. The present inventors found that the ES-like cells have pluripotency as ES cells do since the ES-like cells develop a teratoma when transplanted subcutaneously or otherwise to nude mice, since the ES-like

cells are induced to differentiate into diverse functional cells in vitro, and since the ES-like cells exhibit normal embryogenesis and form extremely diverse tissues, including germ cells, when microinjected into blastocysts, and the like, and developed the present invention.

Accordingly, the present invention relates to the following:

(1) A method of producing pluripotent stem cells, which comprises culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells.

(2) The production method described in (1) above, wherein the medium further contains leukemia inhibitory factor (LIF).

(3) The production method described in (1) or (2) above, wherein the medium further contains at least one of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF).

(4) The production method described in any one of (1) to (3) above, which comprises culturing testis cells in the presence of feeder cells.

(5) The production method described in (1) above, which comprises the following steps:

(Step 1) culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain cultured cells;

(Step 2) culturing the cultured cells obtained in Step 1, using a medium containing leukemia inhibitory factor (LIF) to obtain pluripotent stem cells.

(6) The production method described in (5) above, wherein the medium for Step 1 further contains leukemia inhibitory factor (LIF).

(7) The production method described in (5) or (6) above, wherein the medium for Step 1 further contains at least one of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF).

(8) The production method described in any one of (5) to (7)

above, wherein Step 1 comprises culturing testis cells in the presence of feeder cells.

(9) The production method described in any one of (1) to (8) above, wherein the testis cells are derived from a mammal.

(10) The production method described in (9) above, wherein the mammal is postnatal.

(11) The production method described in (1) above, wherein the pluripotent stem cells are positive for at least any one selected from the group consisting of SSEA-1, Forsman antigen, β1-integrin, α6-integrin, EpCAM, CD9, EE2 and c-kit.

(12) The production method described in (11) above, wherein the pluripotent stem cells are positive for SSEA-1, Forsman antigen, β1-integrin, α6-integrin, EpCAM, CD9, EE2 and c-kit.

(13) A pluripotent stem cell produced by the production method described in any one of (1) to (12) above.

(14) A pluripotent stem cell derived from a testis cell, which is positive for at least any one selected from the group consisting of SSEA-1, Forsman antigen, β1-integrin, α6-integrin, EpCAM, CD9, EE2 and c-kit.

(15) The pluripotent stem cell described in (14) above, which is positive for SSEA-1, Forsman antigen, β1-integrin, α6-integrin, EpCAM, CD9, EE2 and c-kit.

(16) A method of producing a chimeric embryo, which comprises the following steps:

(Step 1) culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells;

(Step 2) introducing the pluripotent stem cells into a host embryo to obtain a chimeric embryo.

(17) A method of producing a chimeric animal (excluding humans), which comprises the following steps:

(Step 1) culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells;

(Step 2) introducing the pluripotent stem cells into a host

embryo to obtain a chimeric embryo;

(Step 3) transferring the chimeric embryo to the uterus of a host animal to obtain a chimeric animal (excluding humans).

(18) A method of producing mesodermal cells, which comprises the following steps:

(Step 1) culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells;

(Step 2) culturing the pluripotent stem cells under mesodermal cell differentiation conditions to obtain mesodermal cells.

(19) The production method of claim 18, wherein the mesodermal cells are any one selected from the group consisting of blood cell lineage cells, vascular lineage cells and myocardial cells.

(20) A method of producing neuronal lineage cells, which comprises the following steps:

(Step 1) culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells;

(Step 2) culturing said pluripotent stem cells under neuronal lineage cell differentiation conditions to obtain neuronal lineage cells.

(21) The method described in (20) above, wherein the neuronal lineage cells are neurons or glial cells.

(22) A composition for producing pluripotent stem cells derived from a testis cell, which contains glial cell derived neurotrophic factor (GDNF) or an equivalent thereto.

(23) The composition described in (22) above, which further contains leukemia inhibitory factor (LIF).

(24) The composition described in (22) or (23) above, which further contains at least one of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF).

Using the production method of the present invention, it is possible to produce pluripotent stem cells such as ES cells and EG cells, which have conventionally been only obtainable

from a prenatal individual (a fertilized egg, an embryo and the like), from a postnatal individual. Using the pluripotent stem cells, it is possible to construct diverse tissues having histocompatibility for autotransplantation, and the pluripotent stem cells are useful in medical fields such as regeneration medicine and gene therapy. The pluripotent stem cells are also useful in the field of biotechnology because they can be used to prepare a genetically modified animal such as a transgenic animal or a knockout animal.

[Best Mode for Carrying out the Invention]

The method of the present invention of producing pluripotent stem cells comprises culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells.

A pluripotent stem cell refers to a cell that can be cultured in vitro, is capable of growing over a long period, has a self-replicating potential, and has the capability of differentiating into all cells constituting a living organism or precursor cells thereof.

Testis cells include all cells constituting the testis, for example, spermatogonial stem cells, spermatogonia, spermatids, spermatogonia, primary spermatocytes, secondary spermatocytes, spermatozoa, Leydig cells, Sertoli cells, interstitial cells, gonocytes, germ cells and the like can be mentioned.

Testis cells can be prepared from the testis by a method known per se. For example, the testis is extirpated, and the extirpated testis is digested with a lytic enzyme such as collagenase, trypsin, and DNase to disperse testis cells (see, for example, Non-patent Reference 1 and the like). The testis cells are washed with culture medium and the like and used to produce the pluripotent stem cells of the present invention.

The testis cells may be cultured before being used to produce the pluripotent stem cells of the present invention. Culture conditions are not subject to limitation; for example,

as described in Non-patent Reference 1, by culturing testis cells obtained by the above-described enzyme treatment in the presence of glial cell derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF) and the like, spermatogonial stem cells may be grown.

The testis cells may also be those obtained by concentrating a fraction of high capability of producing pluripotent stem cells, before being used to produce the pluripotent stem cell of the present invention. As examples of the fraction, spermatogonial stem cells, spermatogonia, gonocytes, germ cells and the like can be mentioned.

As examples of the method of concentration, a method using an antibody that recognizes a cell surface antigen specifically expressed in the cells of the fraction and using a cell sorter, antibody magnetic microbeads and the like, and the like can be mentioned. For example, spermatogonial stem cells can be concentrated with a cell surface antigen such as α6-integrin or c-kit as the indicator (see, for example, Proc Natl Acad Sci USA, 97, 8346-8351, 2001 and the like). Alternatively, it is also possible to concentrate spermatogonial stem cells using a dye of Hoechst and the like (see, for example, Development, 131, 479-487, 2004 and the like).

The testis cell used in the present invention is not subject to limitation, as long as it is derived from an animal. The animal is not subject to limitation, and the animal may be any of a vertebrate and an invertebrate, and is preferably a vertebrate.

As examples of the vertebrate, a mammal, bird, fish, amphibian and reptile can be mentioned. Examples of the mammal include, but are not limited to, laboratory animals such as mice, rats, hamsters, guinea pigs, and rabbits, domestic animals such as pigs, bovines, goat, horses, and sheep, pet animals such as dogs and cats, and primates such as humans, monkeys, orangutans, and chimpanzees. As the bird, chicken,

partridges, ducks, geese, turkeys, ostriches, emus, ostriches, guinea fowls, pigeons and the like can be mentioned.

The vertebrate is preferably a mammal.

Although the mammal may be prenatal or postnatal, it is preferably postnatal.

When a postnatal animal is used, the animal may be any of a neonate, infant, adult, and aged animal, and the animal used is preferably a infant or neonate, more preferably a neonate. When neonates are used, their postnatal ages cannot be the same since they differ depending on the animal species. In the case of mice, for example, they are 0 to 8 days postnatal.

The term "equivalent to glial cell-derived neurotrophic factor (GDNF)" as used herein encompasses GDNF-like compounds such as neurturin, persephin, and artemin, and other compounds exhibiting an action similar to that of glial cell-derived neurotrophic factor (GDNF) or a GDNF-like compound on a GDNF receptor(s) or an co-receptor(s) (for example, antibodies that specifically recognize a GDNF receptor(s) or an co-receptor(s), agonistic compounds to a GDNF receptor(s) or an co-receptor(s), and the like). As such, the receptor(s) or co-receptor(s) include Ret tyrosine kinase and the GDNF-family receptor α:s, respectively.

A GDNF-like compound means a compound that is structurally similar to glial cell-derived neurotrophic factor (GDNF), or that acts like glial cell-derived neurotrophic factor (GDNF) on a receptor or co-receptor thereof. As the GDNF-like compound, neurturin, persephin, artemin and the like, in particular, can be mentioned.

Glial cell-derived neurotrophic factor (GDNF) and GDNF-like compounds are structurally similar to each other; cRet receptor tyrosine kinase acts as a common signal transmission receptor shared by glial cell-derived neurotrophic factor (GDNF), neurturin, persephin, and artemin.

A compound that acts like glial cell-derived neurotrophic factor (GDNF)" means a compound that acts in the same manner as

glial cell-derived neurotrophic factor (GDNF) on a receptor that transmits the signal of glial cell-derived neurotrophic factor (GDNF) or a co-receptor thereof.

"A GDNF receptor" means a substance that binds to glial cell-derived neurotrophic factor (GDNF) or a GDNF-like compound, i.e., a compound capable of transmitting the signal of glial cell-derived neurotrophic factor (GDNF) or a GDNF-like compound. As the "GDNF receptor", cRet receptor tyrosine kinase, which is a receptor that mediates a signal for glial cell-derived neurotrophic factor (GDNF) or GDNF-like compound, in particular, can be mentioned.

"A GDNF co-receptor" means a receptor that does not transmit the signal of glial cell-derived neurotrophic factor (GDNF) or a GDNF-like compound but activates a receptor that transmits the signal of glial cell-derived neurotrophic factor (GDNF) or a GDNF-like compound. These compounds, in particular, are receptors whose members are called the GDNF family receptor α:s (GFRα). These are also associated with signaling receptor complexes for GDNF, persephin, artemin, and neurturin. As receptors of the family, 4 members (GFRα 1 to 4) (Jing, S., et al., Cell, 85, 9-10 (1996); Jing, S. Q., et al., J. Biol. Chem., 272, 33111-33117 (1997); Trean or, J. J., et al., Nature, 382, 80-83 (1996); Subanto, P., et al., Human Molecular Genetics, 6, 1267-1273 (1997)) are already known. These are capable of independently transmitting signals, all of which are essential to ligand binding and cRet activation.

In the production method of the present invention, the concentration of the glial cell derived neurotrophic factor (GDNF) or an equivalent thereto contained in the medium is generally 0.05 ng/ml to 100 mg/ml, for example, 0.5 ng/ml to 100 μg/ml, preferably 0.5 ng/ml to 10 μg/ml, more preferably 0.5 ng/ml to 1 μg/ml, still more preferably 0.5 to 200 ng/ml, still yet more preferably 0.5 to 50 ng/ml, most preferably 2 to 20 ng/ml.

The medium used in the production method of the present

invention preferably further contains leukemia inhibitory factor (LIF).

In the production method of the present invention, when leukemia inhibitory factor (LIF) is contained in the medium, the concentration thereof is generally 10 to 10^6 units/ml, for example, 10 to 10^5 units/ml, preferably 10^2 to 10^4 units/ml, more preferably $3x10^2$ to $5x10^3$ units/ml.

The medium used in the production method of the present invention preferably further contains at least one of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), more preferably both.

In the production method of the present invention, when epidermal growth factor (EGF) is contained in the medium, the concentration thereof is generally 0.05 ng/ml to 100 mg/ml, for example, 0.5 ng/ml to 100 μg/ml, preferably 0.5 ng/ml to 10 μg/ml, more preferably 0.5 ng/ml to 1 μg/ml, still more preferably 0.5 to 200 ng/ml, still yet more preferably 0.5 to 50 ng/ml, most preferably 2 to 30 ng/ml.

In the production method of the present invention, when basic fibroblast growth factor (bFGF) is contained in the medium, the concentration thereof is generally 0.05 ng/ml to 100 mg/ml, for example, 0.5 ng/ml to 100 μg/ml, preferably 0.5 ng/ml to 10 μg/ml, more preferably 0.5 ng/ml to 1 μg/ml, still more preferably 0.5 to 200 ng/ml, still yet more preferably 0.5 to 50 ng/ml, most preferably 2 to 20 ng/ml.

The cytokine that can be contained in the medium in the present invention [glial cell derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) and the like] may be any one derived from an animal, preferably from the above-described mammal without limitation.

As examples of the glial cell derived neurotrophic factor (GDNF), glial cell derived neurotrophic factors (GDNFs) of humans and rats (WO93/06116 pamphlet), mice (see, for example, Gene 203, 2, 149-157, 1997) and the like can be mentioned.

As examples of the leukemia inhibitory factor (LIF), leukemia inhibitory factors (LIFs) of humans (JP-A-1-502985), mice (JP-A-1-502985), sheep (JP-A-4-502554), pigs (JP-A-4-502554), bovines (JP-A-8-154681) and the like can be mentioned.

As examples of the epidermal growth factor (EGF), epidermal growth factors (EGFs) of mice (see, for example, Nature, 257, 325-327, 1975), humans (see, for example, Proc Natl Acad Sci USA, 88, 415, 1991) and the like can be mentioned.

As examples of the basic fibroblast growth factor (bFGF), human bFGF (see, for example, Endocrine Rev., 8, 95, 1987), bovine bFGF (see, for example, Proc. Natl. Acad. Sci. USA, 81, 6963, 1984), mouse bFGF (see, for example, Dev. Biol., 138, 454-463, 1990), rat bFGF (see, for example, Biochem. Biophys. Res. Commun., 157, 256-263, 1988) and the like can be mentioned.

Also, the cytokine comprises a purified naturally occurring, synthetic or recombinant protein, a mutant protein (including insertion, substitution and deletion mutants), a fragment, and a chemically modified derivative thereof, as long as pluripotent stem cells can be acquired when the cytokine is used in the method of the present invention of producing pluripotent stem cells. The cytokine also comprises a protein substantially homologous to the wild type amino acid sequence of each of the above-described cytokines.

The number of amino acids inserted, substituted or deleted in the mutant protein is generally 1 to 20, preferably 1 to 10, more preferably 1 to 5, most preferably 1 or 2.

"Substantially homologous" means that the degree of homology to the wild type amino acid sequence is preferably 70% or more, more preferably 80% or more, still more preferably 90% or more, most preferably 95% or more. The ratio of homology (%) is calculated as the ratio (%) of amino acid residues present in the lesser of two sequences in alignment that are identical amino acids in the sequence to be compared with,

provided that four gaps can be introduced into a length of 100 amino acids to help sequence alignment, as described in the Atlas of Protein Sequence and Structure v.5, p.124, National Biochemical Research Foundation, Washington, D.C. (1972). Also, an optionally chosen protein that can be isolated on the basis of the cross-reactivity to the antibody against each of the above-described cytokines having the wild type amino acid sequence, and a protein encoded by a gene that isolated by hybridization with the gene that encodes the wild type amino acid sequence of each of the above-described cytokines or a gene segment thereof under stringent conditions, are included as the substantially homologous protein.

As examples of the above-described stringent conditions, the hybridization conditions described by Sambrook, J. et al. in "Expression of cloned genes in E. coli" [Molecular Cloning: A laboratory manual (1989), Cold Spring Harbor Laboratory Press, New York, USA, 9. 47-9. 62 and 11.45-11.61]" and the like (for example, hybridization at about 45°C in 6.0xSSC and the like) can be mentioned.

In culturing stem cells such as pluripotent stem cells, it is possible to achieve more stable cultivation of stem cells by using a medium containing a cytokine such as LIF, EGF, or bFGF. Hence, by using a medium containing LIF, EGF, bFGF and the like in the production method of the present invention, it is possible to produce pluripotent stem cells more stably.

The basal medium for the medium used in the production method of the present invention may be any one known per se without limitation; for example, DMEM, EMEM, RPMI-1640, α-MEM, F-12, F-10, M-199, HAM, ATCC-CRCM30, DM-160, DM-201, BME, SFM-101, Fischer, McCoy's 5A, Leibovitz's L-15, RITC80-7, HF-C1, MCDB107, NCTC135, Waymouth's MB752/1, StemPro-34 SFM and the like can be mentioned. A medium modified to suit for ES cell culture and the like may be used, and a mixture of the above-described basal medium may be used.

The medium can contain an additive known per se. The

additive is not subject to limitation; for example, growth factors (for example, insulin and the like), iron sources (for example, transferrin and the like), polyamines (for example, putrescine and the like), minerals (for example, sodium selenate and the like), saccharides (for example, glucose and the like), organic acids (for example, pyruvic acid, lactic acid and the like), serum proteins (for example, albumin and the like), amino acids (for example, L-glutamine and the like), reducing agents (for example, 2-mercaptoethanol and the like), vitamins (for example, ascorbic acid, d-biotin and the like), steroids (for example, β-estradiol, progesterone and the like), antibiotics (for example, streptomycin, penicillin, gentamycin and the like), buffering agents (for example, HEPES and the like) and the like can be mentioned. It is preferable that each of the additives be contained in a concentration range known per se.

Also, the medium can contain a serum. The serum may be any serum derived from an animal without limitation, and it is preferably a serum derived from the above-described mammal (for example, fetal calf serum, human serum and the like). A serum substitute additive [for example, Knockout Serum Replacement (KSR) (manufactured by Invitrogen Company) and the like] may be used. The concentration of serum is not subject to limitation, and it is generally in the range from 0.1 to 30 (v/v)%.

In the production method of the present invention, testis cells may be cultured in the presence of feeder cells. The feeder cells are not subject to limitation; feeder cells known per se for use in culturing pluripotent stem cells such as ES cells and EG cells while maintaining the pluripotency thereof can be used; for example, fibroblasts (mouse embryonic fibroblasts, mouse fibroblast cell line STO and the like) can be mentioned.

The feeder cells are preferably inactivated by a method known per se, for example, radiation (gamma rays and the like), treatment with an anticancer agent (mitomycin C and the like)

and the like.

The cell culture conditions used in the production method of the present invention may be culture conditions in common use in cell culture technology. For example, culture temperature is generally in the range of about 30 to 40°C; preferably about 37°C. The CO_2 concentration is generally in the range of about 1 to 10%, preferably about 5%. Humidity is generally in the range of about 70 to 100%, preferably about 95 to 100%.

Describing in more detail, the method of the present invention of producing pluripotent stem cells is as follows:

Testis cells separated from the testis are suspended in a medium, sown into a cell culture vessel, and cultured (first culture).

Although the cell culture vessel used may be one for use in ordinary cell culture, it is preferable that the vessel be coated with gelatin and the like to promote the adhesion of testis cells to the vessel. The same applies to the vessels used in the cultures that follow.

Although it is possible to produce pluripotent stem cells solely by continuing the first culture, it is preferable to passage cultured cells in the first culture, preferably non-adherent cultured cells (comprising a reasonable number of germ cells), to another cell culture vessel about 6 to 18 hours after the start of the first culture (for example, after overnight culture) (second culture). The passaged cells form colonies on the base of the cell culture vessel, generally within 1 week after passage, although this time varies depending on culture conditions. The colonization can be confirmed using a microscope and the like.

Preferably, generally 5 to 14 days after the start of the second culture, the cells are dispersed by trypsinization and the like, re-suspended in the medium, and further passaged to a new culture plate (third culture). As passage is repeated in the same way, somatic cells of flat shape disappear.

Therefore, after the second or third passage, it is preferable to culture the cells in the presence of feeder cells. The interval of passages and cell dilution rate are determined as appropriate according to culture conditions; for example, an interval of 2 to 5 days and 1 to 1/4 dilution (preferably 1 to 1/2 dilution in the initial stage of culture) can be mentioned. As examples of the interval of passages and cell dilution rate for an established ES-like colony, an interval of 2 to 5 days and 1/4 to 1/10 dilution can be mentioned.

In the above-described culture, the cultured cells form colonies of two different morphologies about 3 to 6 weeks after the start of culture. One group of colonies have a morphology characterized by an intercellular bridge and morula-like structure, and these are colonies of GS cells. The other group of colonies are more densely packed and have a morphology extremely resembling the morphology of colonies of ES cells, and these are colonies of pluripotent stem cells relating to the present invention. Therefore, the colonies of GS cells and the colonies of pluripotent stem cells relating to the present invention can be clearly distinguished visually.

For example, by selectively picking up a colony of pluripotent stem cells under a microscope using a Pasteur pipette and the like, or by limiting dilution and the like, with the above-described morphologies as the indicators, it is possible to isolate pluripotent stem cells. Alternatively, it is possible to isolate the pluripotent stem cells with cell surface markers and the like for the pluripotent stem cells and the like as the indicator, using a cell sorter and the like.

In the method of the present invention of producing pluripotent stem cells, a medium of the same composition may be used throughout the entire process, and a plurality of media of different compositions may be used sequentially. By doing so, it is sometimes possible to grow the pluripotent stem cells more selectively, and to produce the pluripotent stem cells more efficiently.

For example, the medium used for the culture can be changed from the medium used in the initial stage of testis cell culture (designated as medium A) to a medium more suitable for long-term culture of the pluripotent stem cells during the culture.

That is, it is possible to efficiently obtain pluripotent stem cells by culturing testis cells using the medium A to obtain cultured cells, and culturing the cultured cells using the medium B.

The cytokines that can be contained in the medium A are the same as those described above.

Although the medium B may not contain the above-described cytokines [glial cell derived neurotrophic factor (GDNF) or an equivalent thereto, leukemia inhibitory factor (LIF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF)], it preferably contains leukemia inhibitory factor (LIF).

Also, the concentrations of serum that can be contained in the medium A and medium B, respectively, are the same as those described above; the concentration of serum that can be contained in the medium A is preferably 0.1 to 5 (v/v)%, more preferably 0.3 to 3 (v/v)%. The concentration of serum that can be contained in the medium B is preferably 2 to 30 (v/v)%, more preferably 10 to 20 (v/v)%.

Also, the basal media for the medium A and medium B, respectively are the same as those described above; the basal medium for the medium A can be a basal medium suitably used to culture spermatogonial stem cells (for example, StemPro-34 SFM and the like), and the basal medium for the medium B can be a basal medium suitably used to culture ES cells (for example, DMEM and the like).

The additives the medium A or medium B can contain are the same as those described above.

The timing of changing the medium from the medium A to the medium B is difficult to determine definitely because it varies depending on culture conditions and the like; in the

case of mice, for example, the timing is 10 to 120 days, preferably 14 to 40 days, after the start of the first culture.

Furthermore, it is possible to produce pluripotent stem cells at higher efficiency by culturing the cells using a medium of a composition comprising the medium B supplemented with glial cell derived neurotrophic factor (GDNF) or an equivalent thereto at an above-described concentration for about 4 to 40 days just after the medium A was replaced with the medium B.

Such testis cell culture using the medium A and the medium B may be performed in the presence of feeder cells as described above.

The pluripotent stem cells obtained by the production method of the present invention proliferate while maintaining pluripotency generally for 2 months or more, preferably 5 months or more.

In the maintenance and culture of the isolated pluripotent stem cells, the above-described medium B is preferably used.

Whether or not the cells obtained by the production method of the present invention retain pluripotency can be confirmed by a method known per se exemplified below.

For example, the expression of cell surface markers and the like for the cells obtained is analyzed using a flowcytometer and the like. As useful cell surface markers, SSEA-1 (ES cell marker), Forsman antigen (ES cell marker), β1- and α6-integrin (ES and GS cell markers), EpCAM (ES cell and spermatogonia marker), CD9 (ES cell and spermatogonial stem cell marker), EE2 (spermatogonia marker), c-kit (differentiated spermatogonia marker) and the like can be mentioned.

The pluripotent stem cells obtained by the production method of the present invention, for example, mouse-derived pluripotent stem cells, are positive for at least any one selected from the group consisting of SSEA-1, Forsman antigen, β1- and α6-integrin, EpCAM, CD9, EE2 and c-kit, preferably

positive for all. Also, they are preferably weakly positive for Forsman antigen, EE2 and c-kit. Because GS cells are negative for SSEA-1 and Forsman antigen, the pluripotent stem cells obtained by the production method of the present invention are clearly distinguishable from GS cells.

As used herein, "positive" for the expression of a cell surface marker refers to a state wherein the cell surface marker is expressed on the cell surface, and specific binding of a specific antibody for the cell surface marker can be confirmed. "Weakly positive" refers to a state wherein the amount of cell surface marker expressed is relatively weak, a population with less amount of cell surface marker expressed is relatively prevalent, or the ratio of cell population expressing the cell surface marker is relatively small, compared with other cells, and the like.

In pluripotent stem cells of animal species other than mice, the mode of expression of cell surface markers is the same as with mice. However, provided that a marker exists that is not essentially retained by the animal species, considerations of species differences are made, including the exclusion of the marker from the analysis.

It is also possible to confirm whether or not the cells obtained by the production method of the present invention retain pluripotency by measuring the activity of intracellular alkaline phosphatase in the cells by a method known per se. The pluripotent stem cells obtained by the production method of the present invention, like ES cells, are positive for alkaline phosphatase. On the other hand, because GS cells are weakly positive to negative for alkaline phosphatase, the pluripotent stem cells obtained by the production method of the present invention are clearly distinguishable from GS cells.

Alternatively, it is also possible to confirm whether or not the cells obtained by the production method of the present invention retain pluripotency by analyzing the expression of a gene specifically expressed in pluripotent stem cells and the

like by reverse transcription polymerase chain reaction (RT-PCR). For example, in the case of mouse-derived pluripotent stem cells, essential molecules for maintaining undifferentiated ES cells, such as Oct-4, Rex-1 and Nanog can be mentioned as examples of the gene specifically expressed in pluripotent stem cells. The pluripotent stem cells obtained by the production method of the present invention express at least any gene selected from the group consisting of Oct-4, Rex-1 and Nanog, and preferably express all these genes. In GS cells, almost no expression of Nanog is observed; the pluripotent stem cells are clearly distinguishable from GS cells.

It is also possible to confirm the pluripotency of the cells obtained by the production method of the present invention by injecting the cells into the subcutaneous tissue, seminiferous tubule and the like of an immunodeficient animal or an animal with immune tolerance induced therein, and analyzing for the presence or absence of the formation of teratomas. The pluripotent stem cells obtained by the production method of the present invention are capable of forming teratomas; diverse cells differentiated into the three germ layers (for example, nerves, epidermis, muscles, bronchial epithelium, cartilages, bones and the like) are found in the teratomas.

It is also possible to confirm whether or not the cells obtained by the production method of the present invention retain pluripotency by introducing the cells into host embryos, and analyzing for the presence or absence of the birth of a chimeric animal. The pluripotent stem cells obtained by the production method of the present invention are capable of contributing to the normal development of a chimeric animal when introduced into host embryos.

It is also possible to confirm the pluripotency of the cells obtained by the production method of the present invention by applying a method known per se for differentiating ES cells into various functional cells in vitro, and analyzing

the differentiation capacity in vitro of the cells.

For example, the pluripotent stem cells obtained by the production method of the present invention differentiate into mesodermal cells when cultured under mesodermal cell differentiation conditions known per se.

Examples of mesodermal cells include, but are not limited to, blood cell lineage cells, vascular endothelial cells, myocardial cells, osteocytes, chondrocytes, tendon cells, adipocytes, skeletal muscle cells, smooth muscle cells and the like. Preferably, the mesodermal cells are blood cell lineage cells, vascular endothelial cells or myocardial cells.

Examples of the above-described blood cell lineage cells include, but are not limited to, blood cells (for example, CD45-positive cells and the like), erythroblast lineage cells (for example, Ter119-positive cells and the like), myeloid lineage cells [for example, monocyte lineage cells (for example, MAC1-positive cells and the like), neutrophil lineage cells (for example, Gr1-positive cells and the like)] and the like.

As examples of the above-described myocardial cells, MF20-positive cells and the like can be mentioned; as examples of the above-described vascular endothelial cells, CD31-positive cells and the like can be mentioned.

The mesodermal cell differentiation conditions include, but are not limited to, conditions known per se that allow ES cells to differentiate into mesodermal cells; for example, culture in a plate coated with type IV collagen (see, for example, Blood, vol.93, p1253-1263, 1999 and the like), co-culture with feeder cells for inducing mesodermal cell differentiation (for example, stroma cells such as OP9 cells) (see Proc. Natl. Acad. Sci. USA, vol.100, p4018-4023, 2003; Exp. Hematol., vol.22, p979-984; Science, vol.272, 722-724, 1996 and the like) and the like can be mentioned.

When the pluripotent stem cells obtained by the production method of the present invention are differentiated

into blood cell lineage cells or vascular endothelial cells, it is desirable that the pluripotent stem cells be co-cultured with the above-described feeder cells for inducing mesodermal cell differentiation (see, for example, "Proc. Natl. Acad. Sci. USA, vol.100, p4018-4023, 2003", "Exp. Hematol., vol.22, p979-984", "Science, vol.272, 722-724, 1996" and the like).

When the pluripotent stem cells obtained by the production method of the present invention are differentiated into myocardial cells, the pluripotent stem cells are suitably co-cultured with the above-described feeder cells for inducing mesodermal cell differentiation in the presence of SCF (see, for example, Proc. Natl. Acad. Sci. USA, vol.100, p4018-4023, 2003 and the like).

Also, the pluripotent stem cells obtained by the production method of the present invention differentiate into neuronal lineage cells when cultured under neuronal lineage cell differentiation conditions known per se.

As examples of the neuronal lineage cells, neurons (for example, MAP2-positive cells and the like), glial cells (for example, MBP-positive cells and the like), and the like can be mentioned.

The neuronal lineage cell differentiation conditions include, but are not limited to, conditions known per se that allow ES cells to differentiate into neuronal lineage cells; for example, culture using a medium for inducing neuronal differentiation (for example, N2B27 medium) and the like can be mentioned (see, for example, Nature Biotechnology, vol.21, 183-186, 2003 and the like).

The pluripotent stem cells obtained by the production method of the present invention can be cryopreserved semi-permanently, and can be used after thawing and awakening from dormancy as required. The pluripotent stem cells maintain pluripotency even after cryopreservation and thawing. In the cryopreservation, the cells are suspended in a composition for cryopreservation of cells known per se, such as Cell Banker

(manufactured by DIA-IATRON Company), which comprises dimethylsulfoxide and fetal calf serum albumin, and the cells are preserved under conditions of -80 to -200°C, preferably -196°C (in liquid nitrogen).

If the pluripotent stem cells obtained by the production method of the present invention are awaken from dormancy after cryopreservation, the cells are thawed in a solvent according to a conventional method and suspended to yield a cell suspension. The method of thawing is not subject to limitation; for example, thawing can be performed in a 37°C thermostat bath using a DMEM containing 10% fetal calf serum (DMEM/FCS). Specifically, the freezing tube is floated in the thermostat bath, and DMEM/FCS is added drop by drop to the frozen cells to thaw the cells. After the cells are centrifuged and washed, they are re-suspended in the medium.

Even when the pluripotent stem cells once awakened from dormancy are cultured and then again frozen, the pluripotency of the cells is maintained.

Because the pluripotent stem cells obtained by the production method of the present invention are capable of proliferating over a long period while maintaining their pluripotency, it is possible to modify a gene of the pluripotent stem cells by a method known per se, and to produce genetically modified pluripotent stem cells, for example, pluripotent stem cells transfected with a particular exogenous gene, pluripotent stem cells lacking a particular gene, and the like.

As examples of the method of introducing an gene to pluripotent stem cells produced by the method of the present invention, a method comprising introducing a vector constructed to allow the functional expression of a particular gene to spermatogonial stem cells can be mentioned. As the vector, a plasmid vector, a viral vector and the like can be used. Additionally, as the viral vector, retrovirus, adenovirus, lentivirus, herpesvirus, adeno-associated virus, parvovirus,

Semliki forest fever virus, vaccinia virus and the like can be mentioned.

As examples of the method of introducing a vector to pluripotent stem cells, common gene transfection methods such as the calcium phosphate method, the DEAE dextran method, the electroporation method, or the lipofection method can be mentioned. When using a virus as the vector, the virus' genome may be introduced to cells by one of the above-described common gene transfection methods, and the virus' genome can also be introduced to cells by infecting the cells with virus particles.

For selecting genetically modified pluripotent stem cells stably incorporating an particular extraneous gene, a marker gene, simultaneously with a vector, may be introduced to these cells, and the cells may be cultured by a method suitable for the properties of the marker gene. For example, when the marker gene is a gene that confers drug resistance to a selection drug that is lethal to the host cells, the spermatogonial stem cells incorporating a vector may be cultured using a medium supplemented with the drug. As examples of the combination of a drug-resistance-conferring gene and a selection drug, a combination of the neomycin-resistance-conferring gene and neomycin (G418), a combination of the hygromycin-resistance-conferring gene and hygromycin, a combination of the blasticidin-S-resistance-conferring gene and blasticidin S, and the like can be mentioned.

As an example of the method of obtaining pluripotent stem cells lacking a particular gene, homologous recombination using a targeting vector (gene targeting method) can be mentioned. Specifically, pluripotent stem cells lacking a particular gene can be obtained by isolating the chromosome DNA of the particular gene; introducing, to the chromosome of pluripotent stem cells by the homologous recombination method, a DNA strand (targeting vector) having a DNA sequence constructed to destroy the gene by inserting, to an exon portion of the gene, a drug

resistance gene represented by the neomycin resistance gene or the hygromycin resistance gene, a reporter gene represented by *lacZ* (β-galactosidase gene), *cat* (chloramphenicol acetyltransferase gene) and the like to destroy the exon function, by inserting a DNA sequence that terminates gene transcription to the intron portion between exons (for example, polyA addition signal and the like) to prevent the synthesis of complete messenger RNA, and the like; analyzing the thus-obtained cells by Southern hybridization analysis using a DNA sequence in the DNA of a particular gene or a DNA sequence in the vicinity of the DNA as a probe or by a PCR method with primers of the DNA sequence in the targeting vector and a DNA sequence in the vicinity of, but other than, the DNA of the particular gene used to prepare the targeting vector; and selecting pluripotent stem cells lacking the particular gene. Alternatively, the Cre-loxP system, which deletes a particular gene in a tissue-specific or developmental-stage-specific manner, and the like may also be used (Marth, J.D. (1996) Clin. Invest. 97:1999-2002; Wagner, K.U. et al. (1997), Nucleic Acids Res. 25:4323-4330).

The pluripotent stem cells obtained by the production method of the present invention have the capability of differentiating into all somatic cells constituting a living organism; all experimental techniques and methods applicable to ES cells or EG cells can be applied to the pluripotent stem cells; using the pluripotent stem cells, it is possible to produce diverse functional cells, tissues, animals (excluding humans) and the like. Provided that pluripotent stem cells genetically modified by the above-described method are used, it is possible to produce genetically modified diverse functional cells, tissues, animals (excluding humans) and the like.

For example, it is possible to produce the above-described mesodermal cells by culturing the pluripotent stem cells obtained by the production method of the present invention under the above-described mesodermal cell

differentiation conditions.

Also, it is possible to produce the above-described neuronal lineage cells by culturing the pluripotent stem cells obtained by the production method of the present invention under the above-described neuronal lineage cell differentiation conditions.

Additionally, it is possible to produce diverse functional cells by inducing the pluripotent stem cells obtained by the production method of the present invention to differentiate into diverse functional cells using, for example, a method of differentiating ES cells into vascular endothelial cells (Development, vol.125, 1747-1757, 1998), a method of differentiating ES cells into nerve cells (Neuron, vol.28, 31-40, 2000), a method of differentiating ES cells into pigment cells (Development, vol.125, 2915-2923), a method of differentiating ES cells into insulin-producing cells (Proc Natl Acad Sci USA, 97, 11307-11312), a method of differentiating ES cells into ectodermal cells (pamphlet for WO01/088100), a method of producing endodermal cells, ectodermal cells, mesodermal cells, blood cells, endothelial cells, chondrocytes, skeletal muscle cells, smooth muscle cells, myocardial cells, glial cells, neurons, epithelial cells, melanocytes, or keratinocytes by forming an embryoid body of ES cells (Reprod. Fertil. Dev., 10, 31, 1998) and the like.

It is also possible to transfer the pluripotent stem cells obtained by the production method of the present invention to an immunodeficient animal such as a nude mouse, or to an animal with immune tolerance induced therein, to form teratomas, and to isolate diverse functional cells from the teratomas.

Production of an animal (excluding humans) using the pluripotent stem cells relating to the present invention can be performed in accordance with, for example, a method known per se such as a method using a chimeric embryo.

For example, first, a pluripotent stem cell obtained by the production method of the present invention is introduced into a host embryo to obtain a chimeric embryo. The animal species of the "host" is preferably the same as the animal species of the pluripotent stem cell introduced. Examples of the "embryo" include, but are not limited to, blastocysts, 8-cell stage embryos and the like.

An "embryo" can be obtained by mating a female animal that received a superovulation treatment with a hormone preparation (for example, PMSG, which has FSH-like action, and hCG, which has LH action, are used) and the like with a male animal and the like. As methods of introducing a pluripotent stem cell into a host embryo, the microinjection method and aggregation method are known, and any method can be used.

Next, the chimeric embryo is transferred to the uterus of the host animal to obtain a chimeric animal (excluding humans). The host animal is preferably a pseudo-pregnant animal. A pseudo-pregnant animal can be obtained by mating a female animal in the normal sexual cycle with a male animal emasculated by vasoligation and the like. The host animal having the transferred chimeric embryo will become pregnant and bear a chimeric animal (excluding humans).

Furthermore, it is possible to obtain an animal (excluding humans) harboring the gene derived from the pluripotent stem cells by mating the chimeric animal (excluding humans) with a normal animal, and selecting an individual harboring the gene derived from the pluripotent stem cells from among the individuals of next generation (F1). In selecting an animal (excluding humans) harboring a gene derived from pluripotent stem cells, various characters can be used as indicators; for example, body color and coat color are used as the indicators. It is also possible to perform the selection by extracting DNA from a portion of the body and performing Southern blot analysis or PCR assay.

By using the above-described method, for example, it is

possible to obtain an animal (transgenic animal) harboring a particular exogenous gene from pluripotent stem cells transfected with the exogenous gene. Also, from pluripotent stem cells lacking a particular gene, it is possible to obtain a gene-deficient heterozygotous animal. Furthermore, by propagating the gene-deficient heterozygotous animals obtained, it is possible to obtain a gene-deficient homozygotous animal.

The present invention also relates to a composition for producing pluripotent stem cells derived from testis cells, which contains glial cell derived neurotrophic factor (GDNF) or an equivalent thereto. By culturing testis cells by the above-described method using a medium containing the composition, it is possible to obtain pluripotent stem cells derived from the testis cells.

The composition can further contain leukemia inhibitory factor (LIF).

Also, the composition can further contain at least one of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), preferably all.

The composition can further contain a physiologically acceptable carrier, excipient, antiseptic, stabilizer, binder, solubilizer, nonionic surfactant, buffering agent, preservative, antioxidant, the above-described additive, basal medium and the like.

The composition is used in the form of an isotonic aqueous solution or powder and the like, added to the medium used in the production method of the present invention and otherwise. Alternatively, the composition may be a medium used for the production method of the present invention.

The present invention is explained in more detail in the following by referring to Examples, which are not to be construed as limitative.

【Examples】

Materials and Methods

(Cell Culture)

Testis cells were collected from newborn (0-8 days old) DBA/2 mice or transgenic mouse line C57BL6/Tg14(act-EGFP-Osby01) that was bred into DBA/2 background (designated Green) (provided by Dr. M. Okabe, Osaka University). Because these Green mice have the expressed the EGFP gene in substantially all cell types, it is possible to track the cells derived from the mice can be tracked with the fluorescence of EGFP as the indicator.

Testis cells were collected by two-step enzymatic digestion using collagenase (type IV, Sigma) and trypsin (Invitrogen).

That is, the mouse testis was extirpated, the tunica albuginea was removed in PBS, and incubation was performed in Hunks' balanced solution containing 1 mg/ml collagenase (type I) at 37°C for 15 minutes with shaking as appropriate to loosen the seminiferous tubule. After removal of the non-adherent interstitial cells by two times of washing with PBS, incubation was performed in a 0.25% trypsin solution containing 1.4 mg/ml DNase at 37°C for 15 minutes with shaking as appropriate to disassemble the seminiferous tubule. After PBS was added to inactivate the trypsin, pipetting was performed to obtain a cell suspension. This was passed through 20- to 30-μm nylon meshes to remove the undigested cell mass, centrifugation was performed at 600 x g for 5 minutes, and testis cells were recovered.

Testis cells were allocated to a gelatin-coated tissue culture plate. Culture medium for the testis cells was StemPro-34 SFM (Invitrogen) supplemented with StemPro supplement (Invitrogen), 25 μg/ml Insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 6 mg/ml D-(+)-glucose, 30 μg/ml pyruvic acid, 1 μl/ml DL-lactic acid (Sigma), 5 mg/ml bovine albumin (ICN Biomedicals), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, MEM non-essential vitamin solution (Invitrogen), 10^{-4}M ascorbic acid, 10 μg/ml d-biotin, 30 ng/ml β-estradiol, 60 ng/ml progesterone (Sigma), 20

ng/ml mouse epidermal growth factor (EGF: Becton Dickinson), 10 ng/ml basic fibroblast growth factor (bFGF:Becton Dickinson), 10^3 units/ml ESGRO (mouse leukemia inhibitory factor: LIF, Invitrogen), 10 ng/ml recombinant rat GDNF (R&D Systems), and 1 (v/v)% fetal calf serum (JRH Biosciences). The cells were maintained at 37°C in an atmosphere of 5% carbon dioxide in air.

After overnight incubation, the floating cells were passaged to secondary culture plates after vigorous pipetting. Within 1 wk, the passaged cells proliferated, spread on the bottom of the plate, and formed colonies.

Cells were dispersed by trypsin treatment and transferred at intervals of 5-14 days (this interval is called "DIV" for short) to a fresh culture plate (× 1-1/2 dilution) *in vitro*. Colonies grew to the original size in about 10 days, and cells were again passaged (× 1 dilution). From the second or third passage, the cells were maintained on mitomycin C-inactivated mouse embryonic fibroblasts (MEF) and were passaged to new MEF, at 1 to 1/2 fold dilution in the initial stage of cultivation, and at 1 to 1/4 fold dilution thereafter, every 2 to 5 days. Furthermore, the established ES cell-like colony was passaged to new MEF at 1/4 to 1/10 fold dilution, every 2 to 5 days.

After appearance of ES cell like colonies, the cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15 (v/v)% FCS, 0.1 mM 2-mercaptoethanol, 10^3 units/ml ESGRO (mouse leukemia inhibitory factor, Invitrogen) and 10 ng/ml recombinant rat GDNF (R&D Systems), at a final concentration.

Subsequently, the cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15 (v/v)% FCS, 0.1 mM 2-mercaptoethanol, and 10^3 units/ml ESGRO (mouse leukemia inhibitory factor, Invitrogen), at a final concentration.

ES-like cells and GS cells could also be separated by picking up colonies with a Pasteur pipette and the like under a stereoscopic microscope.

Induction of differentiation of cultured cells into blood cell lineage cells in vitro was performed as described in Science, vol.272, p722-724, 1996. That is, by culturing the cultured cells on the OP9 stroma feeder, differentiation into blood cell lineage cells was induced.

Induction of differentiation of cultured cells into myocardial cells in vitro was performed as described in Proc. Natl. Acad. Sci. USA, vol.100, p4018-4023, 2003. That is, by culturing the cultured cells in the presence of SCF on the OP9 stroma feeder, differentiation into myocardial cells was induced.

Induction of differentiation of cultured cells into neurons and glial cells in vitro was performed using N2B27 medium as described in Nature Biotechnology, vol.21, p183-186, 2003. In brief, the cultured cells were plated onto 0.1% gelatin-coated tissue culture plastic at a density of 0.5-1.5 x 10^4 / cm^2 in N2B27 medium. Medium renewed every 2 days. N2B27 is a 1:1 mixture of DMEM/F12 (Sigma) supplemented with modified N2 (25 g/ml insulin, 100 g/ml apo-transferrin, 6 ng/ml progesterone, 16 g/ml putrescine, 30 nM sodium selenite and 50 g/ml bovine serum albumin fraction V (Gibco)) and Neurobasal medium supplemented with B27 (both from Gibco).

Induction of differentiation of cultured cells into myocardial cells in vitro was performed as described in Proc. Natl. Acad. Sci. USA, vol.100, p4018-4023, 2003. That is, by culturing the cultured cells in the presence of SCF on the OP9 stroma feeder, differentiation into myocardial cells was induced.

Induction of differentiation of cultured cells into vascular endothelial cells in vitro was performed as described in Proc. Natl. Acad. Sci. USA, vol.100, p4018-4023, 2003. That is, by culturing the cultured cells on the OP9 stroma feeder, differentiation into vasculo-hematopoietic precursor cells was induced, sorting PECAM-1-positive cells 5 days later, and further culturing the sorted cells on the OP9 stroma feeder,

differentiation into vascular endothelial cells was induced.

ES cells derived from 129svj mice were used.

(Antibodies and staining)

To confirm the properties of cells produced by the production method of the present invention, flowcytometry was performed to examine for the expression of markers for ES cells, spermatogenic cells and the like known per se.

As primary antibodies, rat anti-EpCAM (G8.8), mouse anti-SSEA-1 (MC-480) (Developmental Studies Hybridoma Bank, University of Iowa), rat anti-mouse Forssman antigen (M1/87), rat anti-human α6-integrin (CD49f) (GoH3), biotinylated hamster anti-rat β1-integrin (CD29) (Ha2/5), APC-conjugated rat anti-mouse c-kit (2B8), rat anti-mouse CD9 antibody (BD Biosciences) and rat anti-TDA (EE2; provided by Dr. Y. Nishimune, Osaka University) were used.

APC-conjugated goat anti-rat-IgG (Cedarlane Laboratories), APC-conjugated streptavidin (BD Biosciences), Alexa Fluor 647-conjugated goat anti-rat IgM or Alexa Fluor 633-conjugated goat anti-mouse IgM (Molecular Probes) were used as secondary antibodies. The cell staining was carried out according to Proc Natl Acad Sci USA, vol.96, p5504-5509, 1999. Cells were analyzed with a FACSCalibur system (BD Biosciences).

Immunocytochemistory for functional cells differentiated in vitro was carried out using standard protocols.

Cells were fixed in 4% paraformaldehyde in PBS. As the primary antibodies, anti-MAP2 rabbit polyclonal antibody, anti-MBP mouse polyclonal antibody (Chemicon), mouse anti-myosin heavy chain monoclonal antibody (MF20) and anti-CD31 monoclonal antibody were used. Localization of antigens was visualized by using secondary antibodies conjugated with Cy3.

Alkaline phosphatase staining was carried out according to Nature, vol.352, 809-811, 1991, and Cell, vol.44, 831-838, 1986.

(Transplantation and analysis of recipients)

In the analysis of teratoma formation, approximately 2 ×

10^6 cultured cells were injected subcutaneously into nude mice, and analyzed 3 weeks after transplantation. Formed tissues were fixed in 10% neutral-buffered formalin and processed for paraffin sectioning. Sections were stained with hematoxylin and eosin, and examined under the microscope. All animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of Kyoto University.

(Chimera Formation)

10-15 cultured cells derived from Green mice were injected into the blastocoel of 3.5 dpc blastocysts of C57BL/6 mice. The blastocysts were returned to the uteri of 2.5 dpc pseudopregnant ICR foster mothers. Approximately 70% of the cells retained the euploid karyotype at the time of injection, which significantly influences the chimerism rate or germline transmission after ES cell injection.

Fetal mice at 12.5 dpc were extirpated and examined using a stereoscopic microscope under UV light. Also, frozen sections of the fetal mice were prepared and analyzed for chimerism with the fluorescence of EGFP derived from Green mice using a fluorescent microscope. PI was used as the control stain.

Newborn chimeric mice born by spontaneous delivery were examined using a stereoscopic microscope under UV light.

(RT-PCR)

Expression analysis for Oct-4, HPRT, Rex-1 and Nanog by RT-PCR were carried out according to the descriptions of Science, vol.297, 392-395, 2002; Mol. Cell. Biol., vol.13, 473-486, 1993; Cell, vol.113, 631-642, 2003 and PNAS, vol.100, 14926-14931, 2003.

Results

When neonatal DBA/2 mouse testis cells were cultured in a medium containing GDNF, bFGF, EGF, and LIF, the majority of the colonies had the typical appearance of GS cells, which are characterized by intercellular bridges and a morula-like structure. However, a few colonies (<5%) were remarkably

similar to ES cells (FIG. 1). These colonies were more tightly packed and generally appeared within 3-6 weeks after initiation of the culture.

These ES cell-like colonies grew selectively with an increase in the number thereof when cultured on a mouse fetal fibroblast feeder using Dulbecco's modified Eagles medium supplemented with FCS, 2-mercaptoethanol, mouse leukemia inhibitory factor (LIF), and glial cell derived neurotrophic factor (GDNF). After two to three passages, most colonies in the culture consisted of these ES-like colonies. These ES-like colonies could be maintained with standard ES cell culture conditions (culture on mouse embryonic fibroblast feeder cells using Dulbecco's modified Eagle's medium supplemented with FCS, 2-mercaptoethanol and mouse leukemia inhibitory factor (LIF)). The morphology of the cells did not change as long as the cells were maintained in ES cell culture conditions.

ES-like cells could be propagated in vitro for more than 5 months with 30 passages while maintaining an undifferentiated state. These results were reproducible; because similar cells were obtained in all of 9 experiments from mice with a different genotype (ICR and the like) and ages (0 to 8 days). However, the frequency of ES-like colony formation decreased with age. Interestingly, the above-mentioned ES-like colony did not appear even when completely established GS cells were cultured under ES cell culture conditions.

To examine the phenotype of the ES-like cells, the present inventors established a culture from Green mice. Since these Green mice express the enhanced green fluorescence protein (EGFP) gene ubiquitously, including in spermatogenic cells (enhanced green fluorescent protein), cultured cells can be distinguished from feeder cells under excitation with UV light.

EGFP-positive cells (ES-like cells) out of the above-described cultured cells were analyzed for the expression of surface antigens. As shown in FIGs. 2 (a) to (h), these cells

were positive for SSEA-1 (ES cell marker), β1- and α6-integrin (ES and GS cell marker), EpCAM (ES and spermatogonia cell marker), and CD9 (ES and spermatogonial stem cell marker), and weakly positive for Forssman antigen (ES cell marker), EE2 (spermatogonia marker), and c-kit (differentiated spermatogonia marker).

In contrast, GS cells were completely negative for SSEA-1 and Forssman antigen (FIGs. 3 (a) and (f)), suggesting that ES-like cells are phenotypically distinct from GS cells. In addition, GS cells were positive for β1- and α6-integrin, EpCAM, CD9, EE2 and c-kit (FIGs. 3 (b)-(e), (g) and (h)).

In addition, ES cells were positive for SSEA-1, β1- and α6-integrin, EpCAM, CD9, Forssman antigen and c-kit, and weakly positive for EE2 (FIGs. 4 (a)-(h)).

Testis cells before the start of culture were negative for SSEA-1; about 40% of the population were positive for Forsman antigen (FIG. 5).

The ES-like cells were also strongly positive for alkaline phosphatase, which is characteristic of ES cells (FIG. 6 (a)). On the other hand, GS cells were weakly positive or negative for alkaline phosphatase (FIG. 6 (b), suggesting that the ES-like cells have a distinct phenotype from GS cells.

By a reverse transcriptase-polymerase chain reaction (RT-PCR), it was shown that the ES-like cells expressed molecules essential for maintaining embryonic ES cells such as Oct-4, Rex-1 and Nanog (FIG. 7). These results suggest that the ES-like cells are similar to ES cells in phenotype. On the other hand, present inventors could not detect expression of Nanog in GS cells, suggesting that the ES-like cells have different phenotypes from GS cells (FIG. 7).

To determine whether said ES-like cells can differentiate into other cell lineages in vitro, ES-like cells derived from Green mice were co-cultured with an OP9 stromal feeder layer. The OP9 stromal feeder cells can support differentiation of ES cells to hematopoietic cells. At 10 days from the start of the

coculture, a part of the EGFP positive cells (ES-like cells) differentiated into blood cells (FIG. 8a). The blood cell system cells comprised erythroblasts (Ter119-positive cells), blood cells (CD45-positive cells), myeloid system cells [myeloid precursor cells, monocytic cells (Mac1-positive cells), and neutrophilic cells (Gr1-positive cells)] (FIGs. 8b-c, FIG. 9).

In addition, when the ES-like cells were cultured under the above-mentioned conditions, a part of the ES-like cells differentiated into myocardial cells (MF20-positive cells), vascular endothelial cells (CD31-positive cells), neurons (MAP2-positive cells) and glial cells (MBP-positive cells) (FIGs. 8d-g).

Said ES-like cells were further analyzed in vivo for their differentiation properties by subcutaneous injection of separated cultured cells to nude mice. Transplanted cells gave rise to typical teratomas in all recipients (8/8) by 3 weeks after transplantation. The tumors (teratomas) contained derivatives of the three embryonic germ layers: neuron, epidermis, muscle, bronchial epithelium, cartilage, bone, and the like (FIG. 8h). Similar results were obtained with three clones. In contrast, no obvious tumors developed after subcutaneous transplantation of GS cells or fresh testis cells into nude mice. Therefore, it was shown that the ES-like cells have the characteristic of differentiating into diverse somatic cell lineage in a manner similar to that for ES cells.

Since the ES-like cells originated from testis, their ability to differentiate into germline cells was examined using the spermatogonial transplantation technique (see, for example, JP-A-7-501705). This method allows spermatogonial stem cells to recolonize the empty seminiferous tubules of infertile animals and differentiate into mature sperm. We transplanted the cultured cells into immune-suppressed W mice. These mice are congenitally infertile and have no germ cells. One month after transplantation, all recipient animals (10/10) developed

teratomas in the testis. The seminiferous tubules were disorganized, and no sign of spermatogenesis was found in histological sections. The cell composition found in the teratomas was similar to that of tumors that developed after subcutaneous injection; this shows that the microenvironment of the seminiferous tubule does not influence the differentiation pattern of the cultured cells. In contrast, GS cells produced normal spermatogenesis within 2 months after transplantation when transplanted into the seminiferous tubules.

Additionally, the present inventors microinjected ES-like cells into blastocysts to examine differentiation properties of the ES-like cells in vivo. This is because ES cells colonize in blastocysts and contribute to all cell types in the body, including germline. Ten to fifteen ES-like cells derived from Green mice were injected into 120 B6 blastocysts. After being cultured in vitro for 24 hours, the chimeric embryos were returned to the uteri of 12 pseudo-pregnant recipient mice. At 12.5 days of viviparity, fetuses had developed normally (FIG. 10(a)), and the expression of EGFP was observed in the whole body of each fetus (FIG. 10(b)). Contribution of donor cells was observed in the brain, intestine, heart, liver, neural tube and and other tissues (FIGs. 11 (a)-(f).

Hence, it was shown that the ES-like cells of the present invention have the capability of differentiating into all somatic cells in vivo as well.

(Discussions)

The results of the above mentioned tests revealed the presence of multipotential stem cells in the postnatal testis. The ES-like cells from the testis can be considered the postnatal counterparts of ES/EG cells. EG cells are considered to be the only example of the isolation of multipotent stem cells from primary germ cells. EG cells are obtained from primordial germ cells (PGC), and such cells cannot be isolated from postnatal germ cells, except when cultured after in vivo teratoma formation. However, since the frequency of derivation

of ES-like cells in the present invention was significantly higher than the rate of spontaneous teratoma formation, the ES-like cells are not likely to be derived from teratoma. Therefore, the ability to become multipotent stem cells may persist in neonatal testis. The present inventors propose to name the cells of the present invention multipotent germline stem cells, or mGS cells, to distinguish them from GS cells, which can differentiate only into germline cells.

An important question that arises from this invention is the origin of mGS cells. One possibility is that mGS cells may reflect a transitional population of ES-like cells that persist from the fetal stage. Gonocytes in the testes of newborns have been reported to be heterogenous; pseudopod gonocytes have the capability of colonizing spermatogenesis after spermatogonial transplantation, whereas round gonocytes do not colonize spermatogenesis but undergoes apoptosis in vitro. Because mGS cells differ from GS cells in terms of spermatogonial stem cell activity, mGS and GS cells may have originated from different types of gonocytes.

Another possibility is that multipotentiality may be one of the characteristics of the spermatogonial stem cells. Irrespective of the potentiality, the spermatogonial stem cells are under severe control so as not to cause multilineage differentiation in vivo, and may be restricted towards germline cells.

In fact, teratogenesis is susceptible to environmental influences; teratoma formation can be significantly enhanced in vivo by ectopic transplantation of the fetal genital ridge (~50-fold). In the method of the present invention of producing pluripotent stem cells, the environment in the testis seems to be suppressive on multilineage differentiation because dilution of somatic cells by passage at an early stage after the start of culture is effective in establishing mGS cells.

Interestingly, the acquisition of multipotentiality was concurrent with the loss of spermatogonial stem cell potential.

Despite their testicular origin, mGS cells formed teratomas when transferred in the seminiferous tubules, indicating that this environment was no longer able to support germ cell development. This contrasts with GS cells, which produce spermatogenesis after long term cultivation. The reason for the loss of spermatogonial stem cell potential is presently unknown; however, the present inventors speculate that it may be related to the loss of responsiveness to GDNF during the course of the establishment of mGS cells, as GDNF is an essential factor for promoting the self-renewing division of spermatogonial stem cells in vivo. While further study is necessary to clarify these events, it is also important to state that the loss of spermatogonial stem cell activity does not necessarily indicate the absence of germline competence in mGS cells. Considering that ES and EG cells contribute to the production of germline chimera by blastocyst injection, it may be reasonable to assume that mGS cells also have germline potential. Whether a chimeric animal can give a fetus derived from mGS cells is being studied now.

The derivation of multipotent stem cells from the postnatal testis has important practical value for medicine. The mGS cells produced by the method of the present invention are different from other reported multipotent cells obtained from postnatal animals in terms of morphology, marker expression, and capacity for differentiation. While it is important to study the biology of individual cell types and assess their potential for clinical application, a major advantage of mGS cells is that techniques used to derive specific lineages of cells from ES cells are applicable directly. The derivation of mGS cells has fewer ethical concerns than does the derivation of ES cells because mGS cells can be obtained from postnatal animals without sacrificing the animals. Furthermore, the availability of histocompatible, multipotent tissue for autotransplantation would circumvent immunological problems associated with ES cell-based

technology. In this sense, it will be important to test the possibility of deriving pluripotent stem cells from mature testis and the scope of differentiation capacity thereof, including the effect of imprinting.

Although postnatal male germ cells have been considered to be fully committed to produce sperm, the present invention demonstrates their pluripotentiality and also indicates that testis can serve as a source to derive ES-like stem cells. Together with GS cells, a new stem cell line described here has important implications in understanding the biology of germline and provides a unique tool for biotechnology and medicine.

[Industrial Applicability]

Using the production method of the present invention, it is possible to produce pluripotent stem cells, which have conventionally been only obtainable from fertilized eggs, embryos and the like, from a postnatal individual. Using the pluripotent stem cells, it is possible to construct diverse tissues having histocompatibility for autotransplantation, and the pluripotent stem cells are useful in medical fields such as regeneration medicine and gene therapy. Also, the pluripotent stem cells are useful in the field of biotechnology because they can be used to prepare transgenic animals, knockout animals and the like.

[Brief Description of the Drawings]

[FIG. 1] FIG. 1 presents photographs showing the morphologies of colonies of GS cells and pluripotent stem cells obtained by the production method of the present invention. In each of the panels "a" to "d", the bar in the lower right indicates 50 μm. "a" is a photograph showing a state wherein colonies of GS cells (white triangle) and colonies of the pluripotent stem cells (white arrow) are co-present in the initial stage of cultivation. "b" is a photograph showing the morphology of colonies of the pluripotent stem cells in the initial stage of cultivation. The pluripotent stem cells are more densely packed. "c" is a photograph showing the morphology of colonies

of the completely established pluripotent stem cells. The morphology of the colonies is completely like ES cell colonies. "d" is a photograph showing the morphology of a typical colony of GS cells.

[FIG. 2] FIG. 2 presents histograms showing the expression of cell surface markers for pluripotent stem cells obtained by the production method of the present invention. There is shown the expression of (a) SSEA-1, (b) β1-integrin, (c) α6-integrin, (d) EpCAM, (e) CD9, (f) Forsman antigen, (g) EE2, and (h) c-kit. The ordinate indicates the number of cells; the abscissa indicates the expression of each cell surface marker as relative intensity of fluorescence. The white columns show histograms in cases where the cells were stained without using a primary antibody (negative control); the black columns show histograms in cases where the cells were stained using a primary antibody. The ratio (%) of cells in the gate to the total cell number is (a) 85.14%, (b) 93.72%, (c) 97.98%, (d) 96.36%, (e) 99.11%, (f) 25.38%, (g) 92.29%, and (h) 57.88%, respectively.

[FIG. 3] FIG. 3 presents histograms showing the expression of cell surface markers for GS cells. There is shown the expression of (a) SSEA-1, (b) β1-integrin, (c) α6-integrin, (d) EpCAM, (e) CD9, (f) Forsman antigen, (g) EE2, and (h) c-kit. The ordinate indicates the number of cells; the abscissa indicates the expression of each cell surface marker as relative intensity of fluorescence. The white columns show histograms in cases where the cells were stained without using a primary antibody (negative control); the black columns show histograms in cases where the cells were stained using a primary antibody. The ratio (%) of cells in the gate to the total cell number is (a) 0.67%, (b) 84.83%, (c) 99.70%, (d) 99.20%, (e) 99.11%, (f) 1.72%, (g) 92.78%, and (h) 64.14%, respectively.

[FIG. 4] FIG. 4 presents histograms showing the expression of cell surface markers for ES cells. There is shown the

expression of (a) SSEA-1, (b) β1-integrin, (c) α6-integrin, (d) EpCAM, (e) CD9, (f) Forsman antigen, (g) EE2, and (h) c-kit. The ordinate indicates the number of cells; the abscissa indicates the expression of each cell surface marker as relative intensity of fluorescence. The white columns show histograms in cases where the cells were stained without using a primary antibody (negative control); the black columns show histograms in cases where the cells were stained using a primary antibody. The ratio (%) of cells in the gate to the total cell number is (a) 96.46%, (b) 99.69%, (c) 97.23%, (d) 96.10%, (e) 99.68%, (f) 79.11%, (g) 81.78%, and (h) 93.90%, respectively.

[FIG. 5] FIG. 5 presents histograms showing the expression of cell surface markers for testis cells before the start of cultivation. There is shown the expression of (a) SSEA-1 and (b) Forsman antigen. The ordinate indicates the number of cells; the abscissa indicates the relative intensity of fluorescence. The white columns show histograms in cases where the cells were stained without using a primary antibody (negative control); the black columns show histograms in cases where the cells were stained using a primary antibody. The ratio (%) of cells in the gate to the total cell number is (a) 0.92% and (b) 43.02%, respectively.

[FIG. 6] FIG. 6 shows the results of alkaline phosphatase staining. There are shown (a) a colony of pluripotent stem cells obtained by the production method of the present invention and (b) a colony of GS cells.

[FIG. 7] FIG. 7 shows the results of RT-PCR analysis. The expression of OCT-4, Rex-1, Nanog and HPRT in GS cells (GS) and pluripotent stem cells obtained by the production method of the present invention (mGS) is shown.

[FIG. 8] FIG. 8 shows functional cells differentiated from pluripotent stem cells obtained by the production method of the present invention: (a) is a figure showing the blood cell lineage cells occurred on OP9 cells, (b) is a figure showing

the results of flowcytometry analysis of the expression of cell surface markers for blood cell lineage cells differentiated in vitro, wherein the ordinate indicates the expression of cell surface markers (negative control, Ter119, CD45 from the left); the abscissa indicates forward scattering, (c) is a figure showing the results of Giemsa staining of blood cell lineage cells differentiated in vitro, wherein the triangle indicates myeloid progenitor, and the arrow indicates erythroblast, (d) is a figure showing the myocardial cells differentiated in vitro (MF20 staining), (e) is a figure showing vascular endothelial cells differentiated in vitro (CD31 staining), (f) is a figure showing neurons differentiated in vitro (MAP staining), (g) is a figure showing glial cells differentiated in vitro (MBP staining), and (h) is a figure showing the results of a histological observation of teratomas.

[FIG. 9] FIG. 9 shows the results of flowcytometry analysis. There are shown the analytical results for (a) negative control, (b) Ter119, (c) CD45, and (d) Mac1/Gr1 (stained with Mix). In (a) to (d), in each upper dot plot, the ordinate indicates the expression of each cell surface marker; the abscissa indicates the expression of EGFP as relative intensity of fluorescence, respectively. In (a) to (d), each table on the bottom shows the number of plots (events), the ratio (% gate) to the number of all surviving cells (% gate), and the ratio (% entire) to the number of all cells in each of the four separate gates (upper left, upper right, lower left, lower right).

[FIG. 10] FIG. 10 shows an enlarged view of a mouse fetus. There are shown observation under (a) visible light and (b) UV light. The left side of both (a) and (b) shows a fetus derived from the litter of a normal embryo, and the right side shows a fetus devired from a chimeric embryo.

[FIG. 11] FIG. 11 shows the results of a histological examination of a frozen section of a chimeric mouse fetus using a fluorescent microscope, wherein (a) brain, (b) heart, (c)

upper portion of the neural tube, (d) intestine, (e) liver and (f) lower portion of the neural tube are respectively shown.

[FIG. 12] FIG. 12 shows the results of an examination of a newborn chimeric mouse pup under UV light.

【Document】 Drawings

FIG. 1

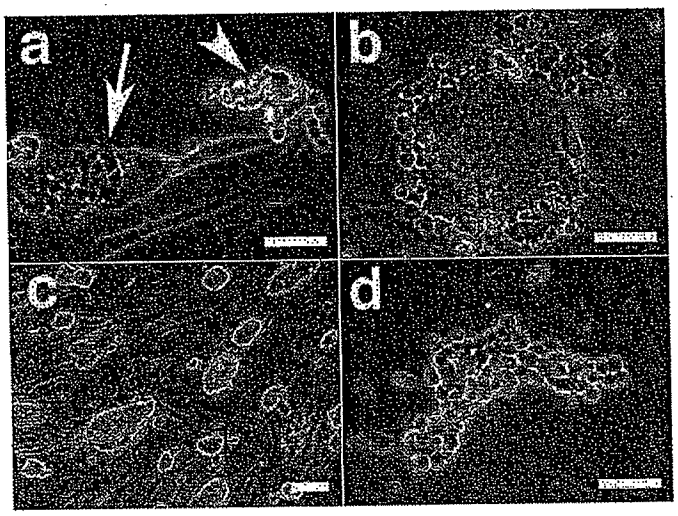
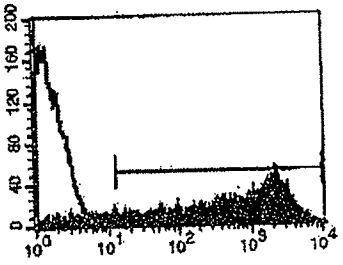


FIG. 2

(a)



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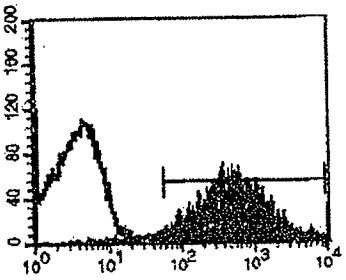
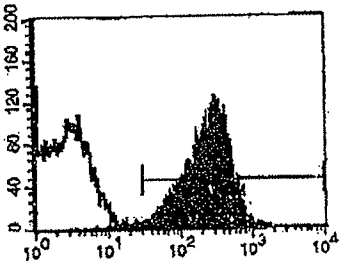


(c)



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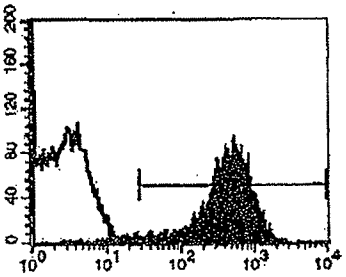

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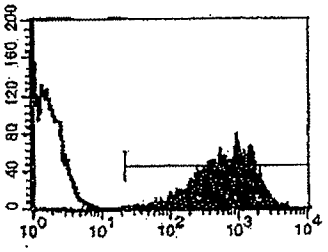

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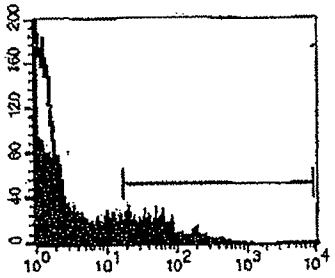

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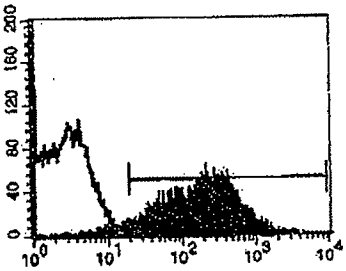

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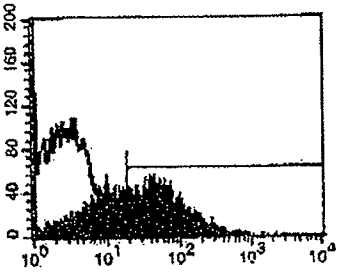
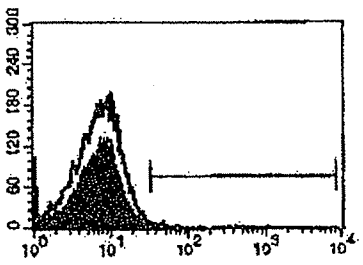


FIG. 3

(a)



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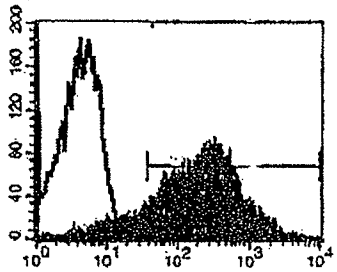

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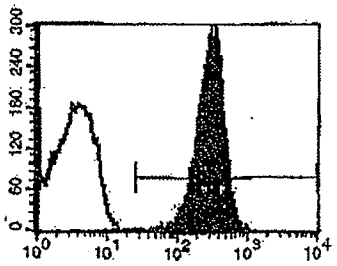

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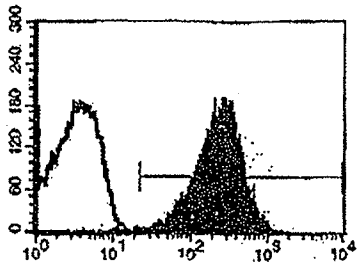

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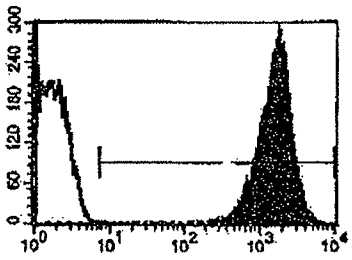

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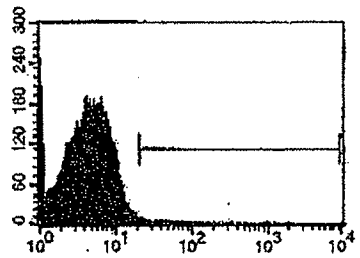

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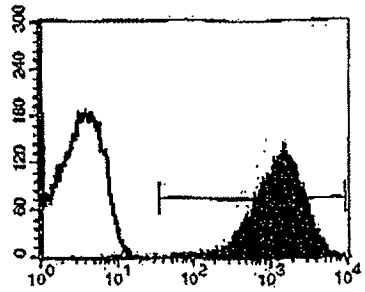

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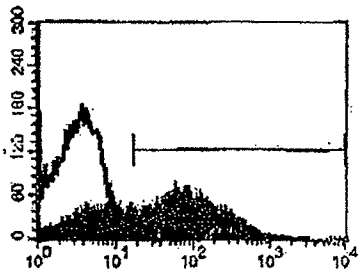
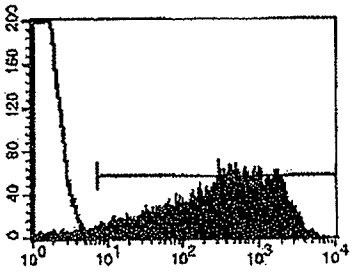


FIG. 4

(a)



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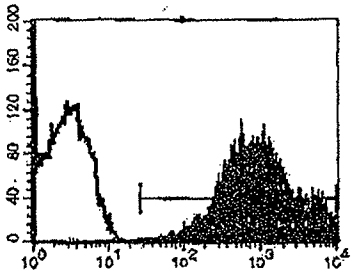

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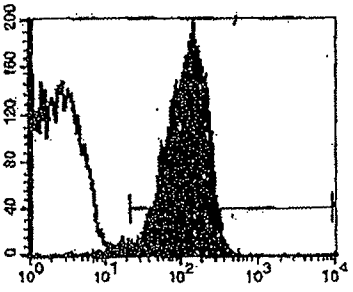

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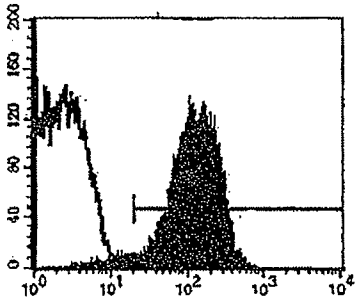

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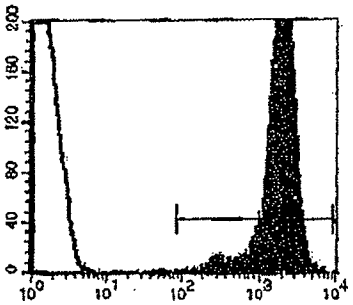

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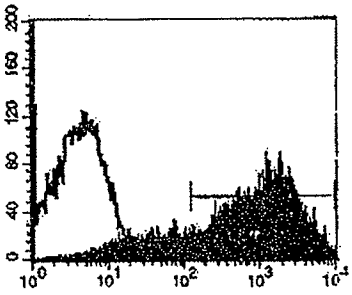

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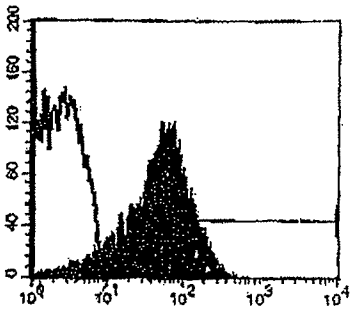

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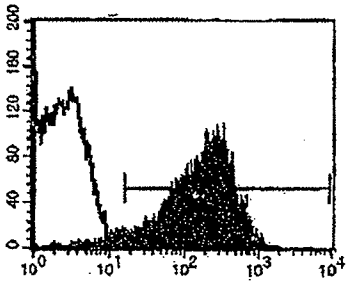
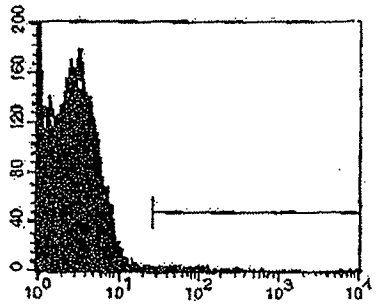


FIG. 5

(a)


0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4

(b)

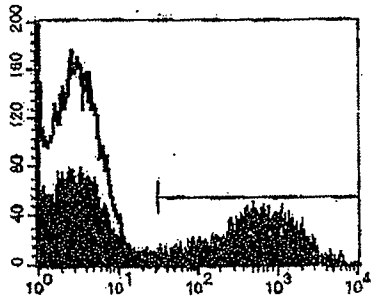
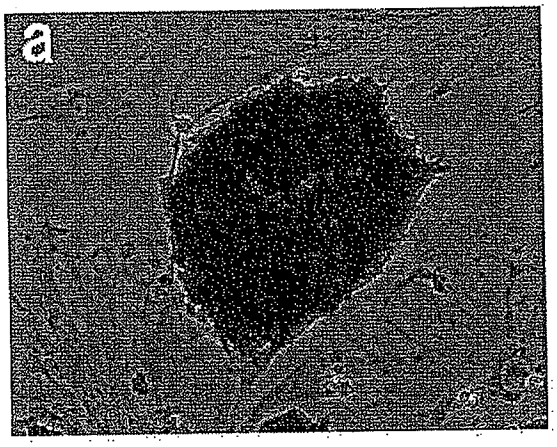

0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4

FIG. 6


a

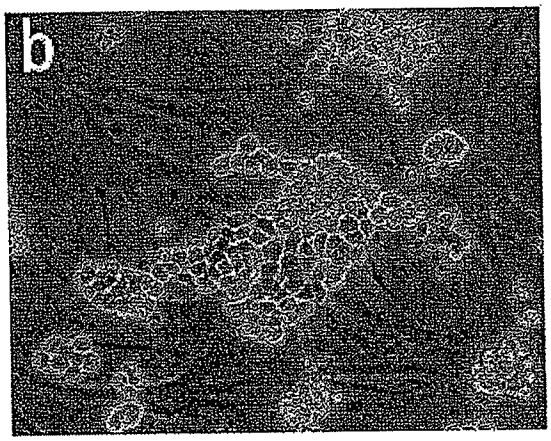

b

FIG. 7

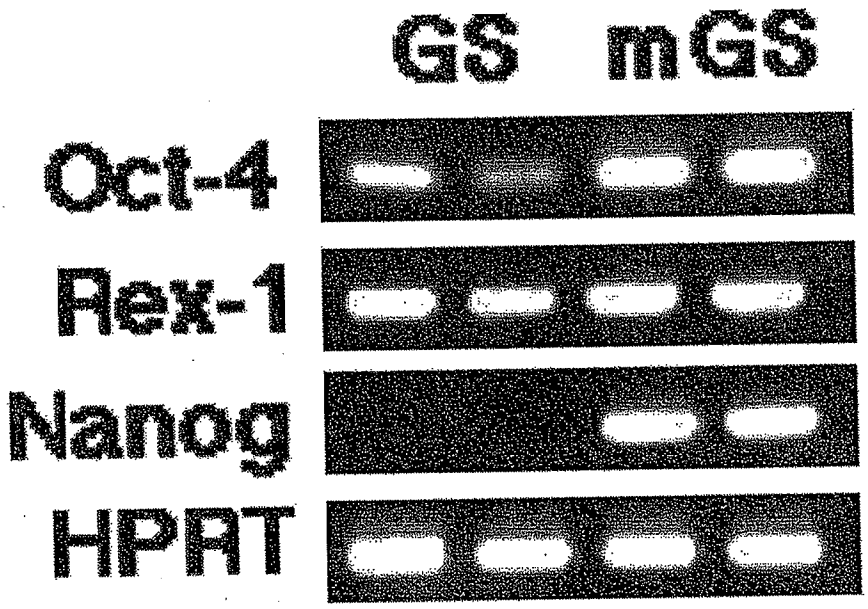


FIG. 8

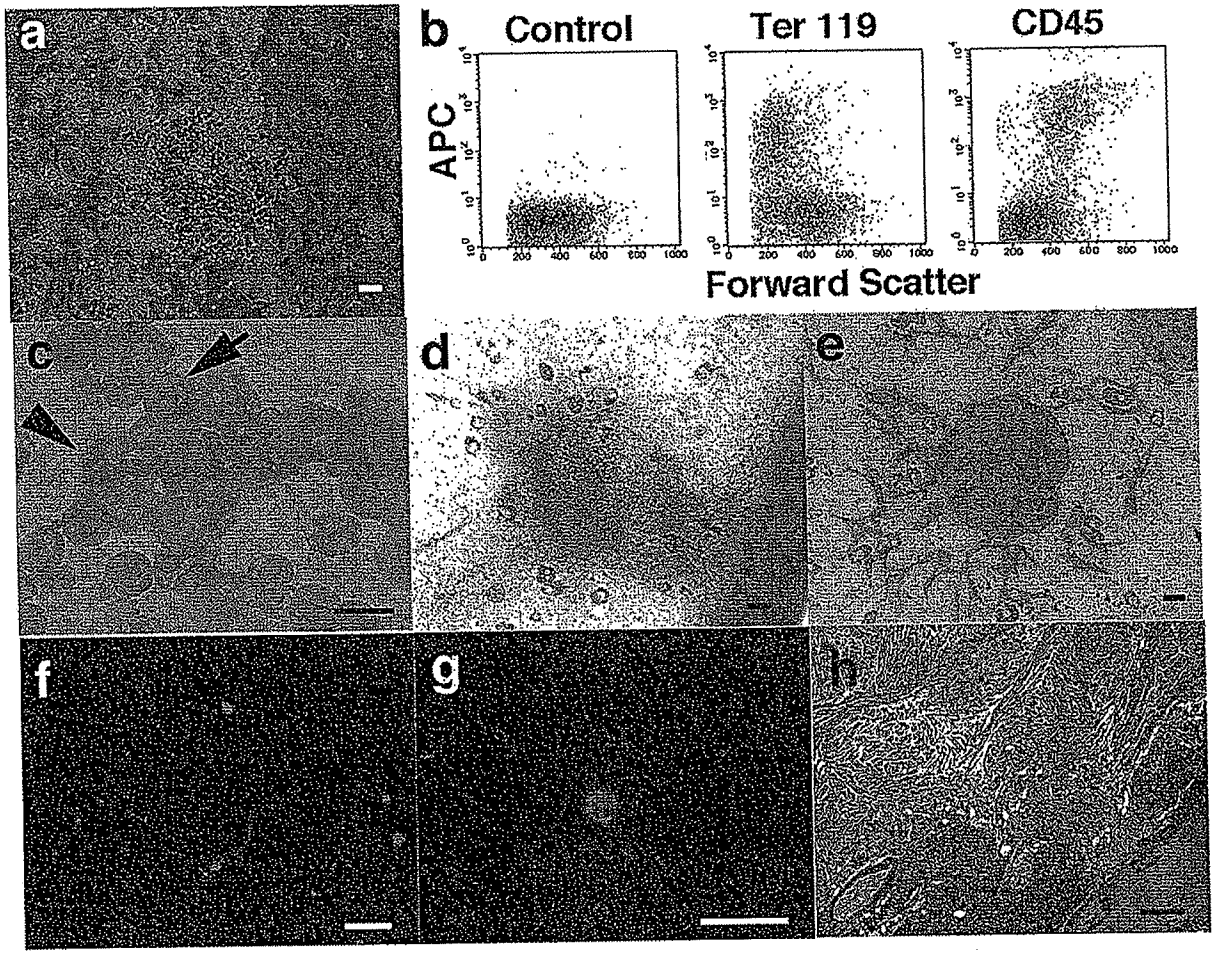
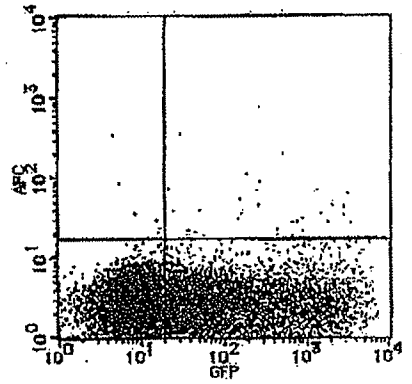


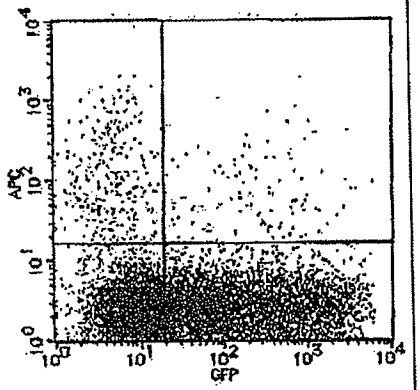
FIG. 9

(a)



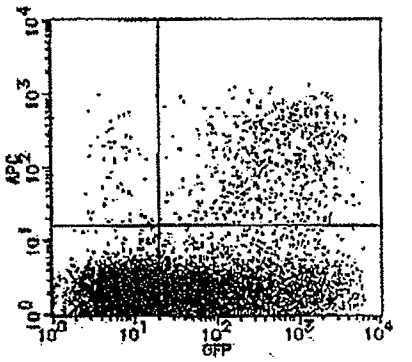
4 separate gates	events	% gate	% entire
upper left	6	0.06	0.04
upper right	35	0.35	0.21
lower left	4884	48.84	28.69
lower right	5075	50.75	29.81

(b)



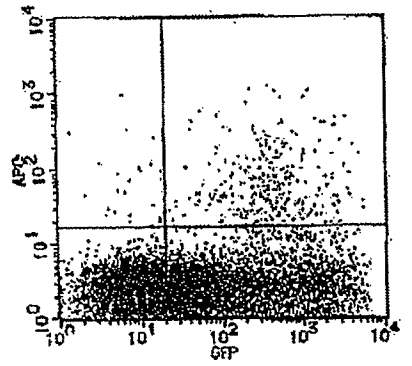
4 separate gates	events	% gate	% entire
upper left	302	3.02	1.61
upper right	120	1.2	0.64
lower left	4653	46.53	24.83
lower right	4925	49.25	26.28

(c)



4 separate gates	events	% gate	% entire
upper left	58	0.58	0.32
upper right	631	6.31	3.45
lower left	5073	50.73	27.78
lower right	4238	42.38	23.2

(d)



4 separate gates	events	% gate	% entire
upper left	23	0.23	0.13
upper right	274	2.74	1.5
lower left	5172	51.72	28.39
lower right	4531	45.31	24.67

FIG. 10

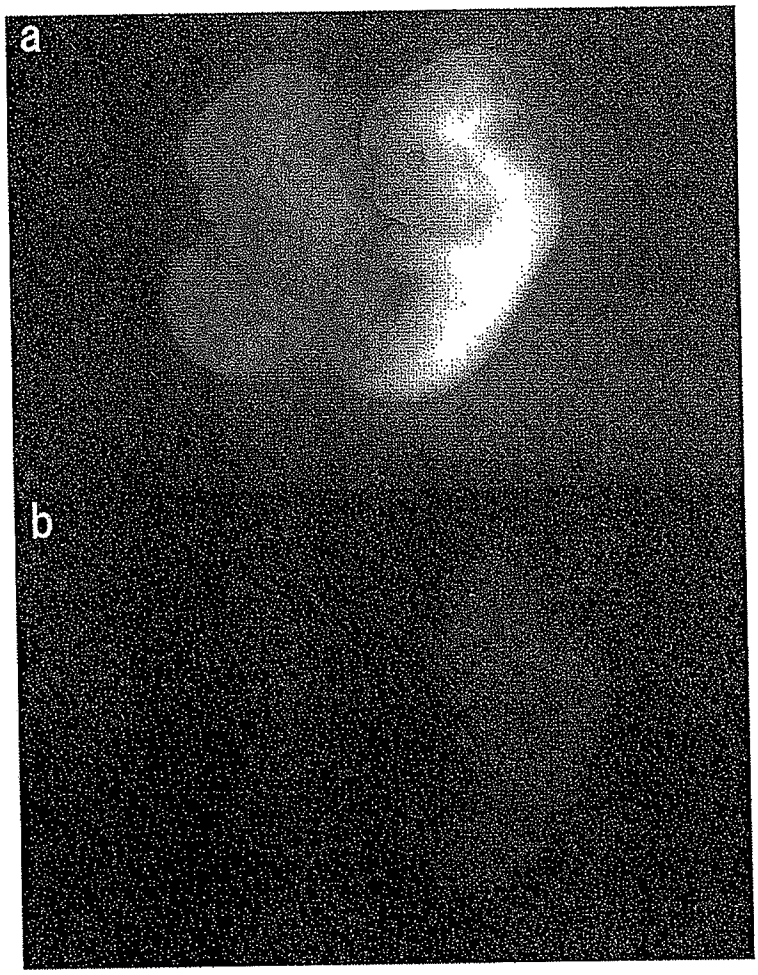


FIG. 11

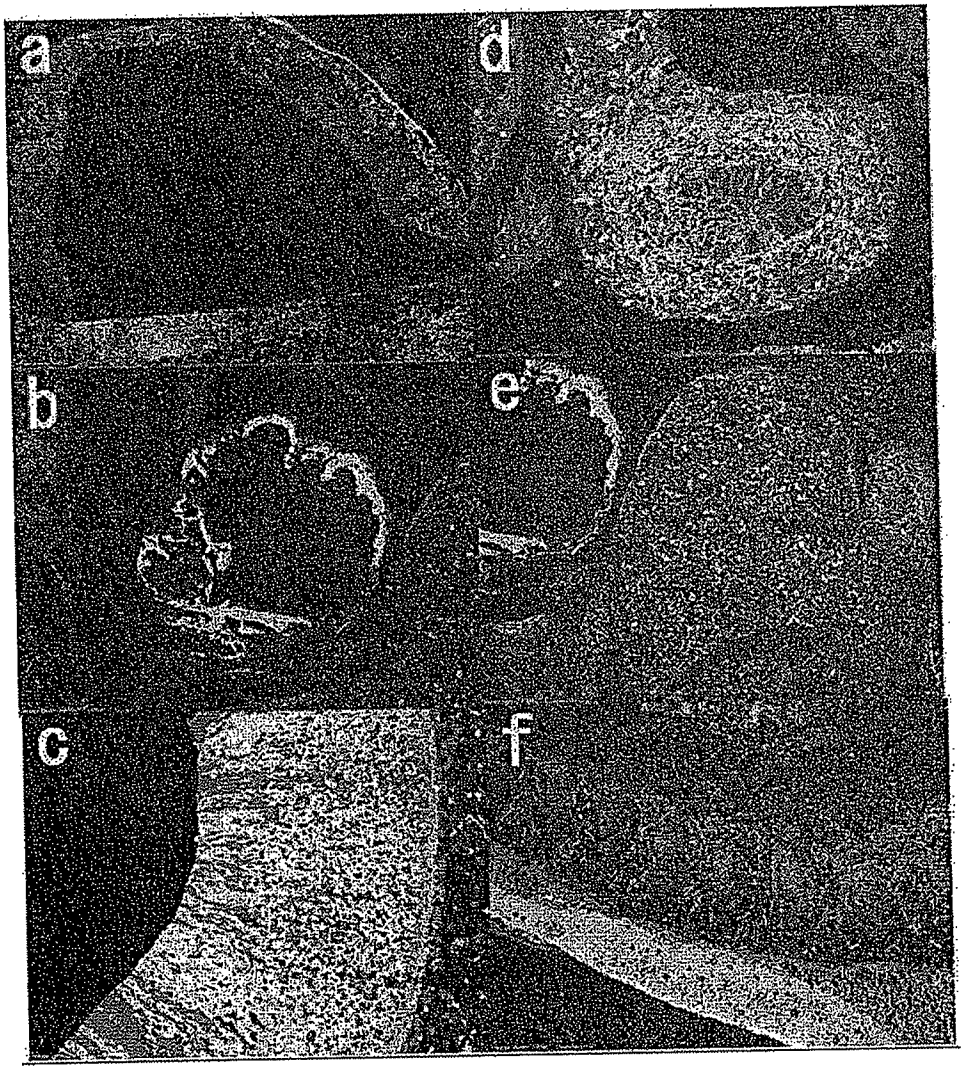


FIG. 12

【Document】 Abstract

【Summary】

【Problem】 Provision of a novel method of producing pluripotent stem cells from a postnatal individual.

【Solving Means】 The present invention provides a method of producing pluripotent stem cells, which comprises culturing testis cells using a medium containing glial cell derived neurotrophic factor (GDNF) or an equivalent thereto to obtain pluripotent stem cells. The medium can further contain leukemia inhibitory factor (LIF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and the like. Using the production method of the present invention, it is possible to produce pluripotent stem cells, which have conventionally been only obtainable from fertilized eggs, embryos and the like, from a postnatal individual. Using the pluripotent stem cells, it is possible to construct diverse tissues having histocompatibility for autotransplantation, and the pluripotent stem cells are useful in medical fields such as regeneration medicine and gene therapy. Also, the pluripotent stem cells are useful in the field of biotechnology because they can be used to prepare transgenic animals, knockout animals and the like.

【Main Drawing】 None